

Article

Culture Media and Sampling Collection Method for *Aspergillus* spp. Assessment: Tackling the Gap between Recommendations and the Scientific Evidence

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Abstract: Culturing is still the most widely used method for determining fungal growth. Thus, is important to identify the most suitable culture media to assess *Aspergillus* spp. The aim of this study was to analyze data obtained from previous studies, aiming at identifying the most suitable culture media (malt extract agar (MEA) or dichloran-glycerol agar (DG18) to assess *Aspergillus* spp. isolation and growth. This study was conducted by using environmental samples ($n = 1153$). Most of the active sampling methods (air samples) were impacted directly onto both culture media. As for passive sampling methods, fungi were extracted from environmental matrices inoculated onto both media. Overall, total *Aspergillus* counts were higher in MEA ($n = 617$, 53.5%) than in DG18 ($n = 536$, 46.5%). Regarding *Aspergillus* sections, significant associations were detected with the media ($\chi^2(7) = 241.118$, $p < 0.001$), the sampling approach ($p < 0.001$, 95% CI = (0.3×10^{-4})), and the indoor environment ($p < 0.001$, 95% CI = (0.3×10^{-4})). As such, sampling approach and the culture media should be accurately selected when dealing with *Aspergillus* spp. exposure assessment.

Keywords: exposure; MEA; DG18; sampling; indoor; *Aspergillus* spp.



Citation: Viegas, C.; Dias, M.; Carolino, E.; Sabino, R. Culture Media and Sampling Collection Method for *Aspergillus* spp. Assessment: Tackling the Gap between Recommendations and the Scientific Evidence.

Atmosphere **2021**, *12*, 23. <https://dx.doi.org/10.3390/atmos12010023>

Received: 4 October 2020

Accepted: 24 December 2020

Published: 26 December 2020

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1. Introduction

Airborne microorganisms are able to spread into most of our living environments, including indoor [1]. Indoor exposure to high concentrations of microorganisms has been recognized as one of the main transmission routes for infectious diseases [2,3], being responsible for various types of health effects through inhalation and ingestion. Additionally, and besides infections, human exposure to bioaerosols is associated with a wide range of respiratory health problems, due to exposure in different occupational environments [4–6]. In what concerns bioaerosols' health effects, fungi play an important role. High levels of fungal particles are found in different occupational environments (animal production and waste industries, among others) [4–6], and their workers are at increased risk for respiratory diseases [7].

The genus *Aspergillus* is known to be one of the most frequently airborne fungi found in indoor and outdoor environments worldwide [8–16]. More recently, due to molecular identification, the genus has been subdivided into 22 different sections, including the clinically relevant species *Aspergilli*, *Fumigati*, *Circumdati*, *Terrei*, *Nidulantes*, *Ornati*, *Warcupi*, *Candidi*, *Restricti*, *Usti*, and *Flavi* [12]. Sections/species from *Aspergillus* genus are found in several habitats with different roles. In fact, they can act as decomposers of organic

matter (for instance, *Aspergillus* section *Nigri*), mycotoxin producers (*Aspergillus* section *Flavi*, among others), and often as human pathogens (*Aspergillus* section *Fumigati*). The role as human pathogens can occur particularly in occupational environments, such as libraries and archives, bakeries, waste-sorting facilities, composting plants, sawmills, animal production, and other agricultural environments [8–16]. *Aspergillus fumigatus* is the species more frequently associated with respiratory symptoms, especially due to the small size of the conidia and other virulence factors, such as gliotoxin production, associated with this species. However, besides the infection ability, risk assessment should consider the toxigenic potential of species belonging to *Aspergillus* genus [17]. Thus, species or strains belonging to sections *Circumdati*, *Flavi*, *Nigri*, and *Nidulantes* must be considered in the risk assessment, since they include species that produce mycotoxins [7,18]. Another topic of concern in specific occupational environments, such as sawmills [14,19], is the exposure to high levels of azole-resistant *Aspergillus* isolates, which may increase the risk of infection, enhancing the difficulty of its clinical management [20–22].

Several sampling methods can be used to perform the *Aspergillus* assessment, as was already reported [12,15,17,23]. Active sampling methods (air sampling), although the most used method in sampling campaigns, can only reflect the load from a shorter period of time (mostly minutes). On the contrary, passive methods (such as surface swabs or settled dust) allow for the determination of the contamination levels from an increased period of time (weeks to several months) [13]. Thus, the sampling approach should comprise more than one sampling method, to allow a wider characterization of the fungal contamination and a more accurate exposure assessment [13,15].

Regarding the analysis of the collected samples, the culture method for the determination of fungal growth is still the most widely used. Traditional quantification of fungi is based on the determination of the number and type of colony forming units (CFU), providing quantitative and qualitative data on viable and culturable fungi [24,25]. Furthermore, counting culturable microorganisms not only allows a quantitative assessment (load or contamination), but also a qualitative analyses of the exposure by identifying the isolated fungi, since not all fungi pose the same hazard. After culture, fungi may be identified to the genus or species level by morphological criteria and microscopy identification [24,25].

Malt extract agar (MEA) is undoubtedly the culture medium most frequently recommended [26–29] and used in aerobiological studies, given the wide range of fungal genera found in the air. It is a nutrient-rich medium for the cultivation of environmental fungi [23,28]. MEA is particularly suitable for yeasts and molds, as it contains a high concentration of maltose and saccharides as energy sources. The acidic pH is optimum for fungal growth, whilst restricting bacterial growth [23,28]. Nevertheless, there are several other efficient culture media, such as DG18 (dichloran-glycerol Agar), firstly used for the enumeration of xerophilic molds and osmophilic yeasts. DG18 contains glycerol at 16% (*w/w*), which lowers the water activity (*aW*) from 0.999 to 0.95 [27]. Glycerol was chosen because of the advantages regarding culture of a wider range of xerophilic fungi, over sodium chloride and sugars, which have traditionally been used to formulate media of reduced *aW* [27]. Various studies have indicated this medium to be a better alternative for colony counting and to obtain higher diversity of genera, since it contains dichloran, which inhibits spreading of fungi belonging to the Mucorales order, such as *Rhizopus* and *Mucor* genera [13–16,30,31], and restricts the colony size of other genera. This restrictive characteristic makes the medium especially suitable for enumeration because it allows for the unobscured growth of organisms that ordinarily form small colonies [27,32]. In summary, MEA allows fast-growing fungal species to grow on its surface, especially due to high sugar content and water activity, while DG18 allows the identification of a more diverse fungal flora, but excludes fungi requiring high water activity to grow [27,32].

In highly contaminated environments, genera with a higher growth rate might inhibit the growth of *Aspergillus*, and, therefore, culture based-methods combined with molecular tools seems to be the best approach to obtain the data necessary to attain legal and scientific criteria [12,14–17,33]. Refined molecular tools allow for the rapid identification of fungi

in air samples (for instance by high-throughput sequencing) and are being increasingly used to obtain information regarding the microbial biodiversity present in different indoor and occupational environments [13,33]. Nevertheless, it is also necessary to assess the viability of the microorganisms, since it affects biological mechanisms, such as the inflammatory and cytotoxic responses [17,34]. This means that the inflammatory potential of one environmental sample (air, settled dust, etc.) may differ depending on its microbial composition and the biological activity of the microorganisms in the sample [16,17,35,36]. This aspect underlines the importance of applying culture-based methods in exposure assessments studies [12,16,17,36], in parallel with molecular tools to overcome limitations of culture-based methods already reported [25].

To the authors' knowledge, there are no studies regarding the evaluation of which is the most suitable culture media to assess *Aspergillus* spp. on environmental samples from different indoor environments in what concerns the fungal counts and diversity of sections. This study aimed to analyze data from previous studies, in order to identify the most suitable culture media between MEA or DG18 to assess *Aspergillus* spp. exposure. We aimed to design the most appropriate strategy on samples' culture, depending on the applied sampling method and on the studied indoor environment.

2. Materials and Methods

2.1. Environmental Samples

This study was conducted by using environmental samples ($n = 1153$) collected from January of 2018 to March 2020 in several indoor and occupational environments located in the Lisbon district (Table 1). The environmental samples were collected in the scope of enlarged financed studies focused on the assessment of occupational exposure to microbiologic agents, with emphasis in the workplaces' fungal contamination. Different companies were called to participate in the study, depending on each project goal. Workplaces enrolled in this study were selected in order to obtain the most critical scenario regarding occupational exposure to fungi [11–16].

Different sampling approaches were applied, and the ones where *Aspergillus* sections were detected and identified are listed in Table 1.

2.2. Sampling and Characterization of Viable Bioburden

Most of the active sampling methods (air samples) were impacted directly onto the culture media. Regarding passive sampling methods, fungal contamination was extracted from the environmental matrix with 0.1% TweenTM 80 saline solution (NaCl 0.9%) for 30 min at 250 rpm on an orbital laboratory shaker (Edmund Bühler SM-30, Hechingen, Germany), and 150 μ L of each wash suspension was inoculated [13–16]. These laboratory procedures were followed since 2018, allowing the feed and update of an algorithm developed to assess occupational exposure to *Aspergillus* genus.

In all the collected samples, two different culture media were used in order to enhance the sensitivity for fungal growth: MEA supplemented with chloramphenicol (0.05%) and DG18. Sampling collection method and laboratory processing are further presented in more detail.

Regarding active sampling methods, indoor air samples in each assessed area/workplace, of a range of different volumes of air (50 to 250 L), were collected by impaction, with a flow rate of 140 L/min (Millipore air Tester, Millipore, Billerica, MA, USA), onto each plate, according to manufacturer's instructions [13]. In some specific cases (bakeries), air samples of 300 L were collected by using the impinger Coriolis μ air sampler (Bertin Technologies) with a flow rate of 300 L/min, onto 10 mL sterile phosphate-buffered saline (PBS) with 0.05% Triton X-100 to be applied for culture and molecular methods [37]. In the firefighters' ambulances project, the Andersen six-stage sampler with six stages and different impaction velocities of 24–1.1-m/s. were also applied. Sampler stages have cutoff sizes of 0.65, 1.1, 2.1, 3.3, 4.7, and 7.0 mm.

Table 1. Indoor and occupational environments assessed and applied sampling approaches.

Indoor/Occupational Environment *	Sampling Approaches	n (%)
Bakeries (n = 91)	EDC	10 (11.0%)
	Air impaction	28 (30.8%)
	Air impingement	6 (6.6%)
	Filter air sampler	5 (5.5%)
	Surface swab	20 (22.0%)
Taxi (n = 17)	HVAC filter	17 (100%)
Veterinary clinics (n = 6)	Air impaction	6 (100%)
Dairies (n = 29)	Air impaction	29 (100%)
Dwellings (n = 184)	Air impaction	51 (27.7%)
	EDC	133 (72.3%)
Elderly care centers (n = 11)	Air impaction	11 (100%)
Elementary school (n = 2)	EDC	2 (100%)
College indoors (n = 17)	Air impaction	13 (76.5%)
	Surface swab	4 (23.5%)
Firefighters' ambulances (n = 16)	Air impaction	9 (56.3%)
	Settled dust	4 (25.0%)
	Surface swab	3 (18.8%)
Healthcare facilities (n = 231)	Air impaction	136 (58.9%)
	HVAC filter	6 (2.6%)
	EDC	20 (8.7%)
	Settled dust	16 (6.9%)
	Surface swab	11 (4.8%)
	Vacuuming bag	3 (1.3%)
	Ventilation grid–swab	39 (16.9%)
Sawmills (n = 10)	Filter air sampler	10 (100%)
Swine (n = 26)	Air impaction	22 (84.6%)
	Surface swab	4 (15.4%)
Thermal baths (n = 17)	Air impaction	15 (88.2%)
	EDC	2 (11.8%)
Waste-sorting plant (n = 488)	HVAC filter	8 (1.6%)
	Filter air sampler	75 (15.4%)
	FRPD	280 (58.0%)
	MPG	125 (25.6%)

EDC—electrostatic dust collector; FRPD—filtering respiratory protection devices; HVAC—heating, ventilation, and air-conditioning; MPG—mechanic protection gloves. * Adopted from References [11–16,37].

In two different occupational environments (bakeries and sawmills), samples were collected through a different active sampling method (filtration). In the bakeries, samples were collected in the worker breathing zone, using portable SKC Sidekick sampling pumps connected to an IOM sampler SKC, Ltd., Dorset, UK, containing 25 mm Whatman GF/A glass microfiber filters (pre-sterilized by autoclaving at a standard temperature and pressure, meaning 121 °C for at least 30 min by using saturated steam under at least 15 psi of pressure) [14,15]. After gravimetric analysis, each filter was extracted in sterile 10 mL deionized water with 0.05% Tween80™ (Sigma-Aldrich, Dorset, UK) at 250 rpm for 1 min, and then a volume of 3.8 mL of sterile glycerol was added, and the solution was extracted again for 1 min at 250 rpm, then stored at −80 °C until the microbial analysis [14,15]. In sawmills, samples were collected with 37 mm conical inhalable sampling (CIS) cassettes with a conical inlet hole of 8 mm (Casella Solutions, Kempston, UK), loaded with a polycarbonate filter (pore size 1 µm) and using an airflow of 3.5 L/min. Exposed filters were transferred to 15 mL tubes containing 5 mL PBS with 0.1% BSA and sonicated for 5 min, followed by shaking for 25 min at 500 rpm. The suspension was poured into a

new tube, and the extraction process repeated with 2 mL PBS–BSA before the suspensions were pooled together. Aliquots of 1 mL of the sample suspensions were stored at -20°C , in a refrigerator, until analysis [14].

Concerning passive sampling methods, surface samples were collected by swabbing indoor sites with a $10\text{ cm} \times 10\text{ cm}$ square stencil, which was disinfected with a 70% alcohol solution between samplings. Surface swab samples were extracted and plated onto both media [13].

Pieces of heating, ventilation, and air-conditioning (HVAC) filters from different indoor environments, filtering respiratory protection devices (FRPD), mechanical protection gloves (MPG), and vacuuming bags with 2 cm^2 ($1.4\text{ cm} \times 1.4\text{ cm}$) were collected and kept refrigerated at 4°C , before analysis; then they were washed, and the extracts were seeded on both media [16].

Each electrostatic dust collector (EDC) having a surface exposure area of 0.0209 m^2 ($19\text{ cm} \times 11\text{ cm}$) was placed at a minimum 0.93 m above floor level, and dust was allowed to settle for 13 to 16 days. Each EDC cloth was washed, and the extracts were also seeded in MEA and DG18 [15].

Settled dust samples were weighted and washed in a ratio of 1 g (dust) per 9.1 mL of NaCl 0.9% with 0.05% Tween 80 (10 μL) for 60 min at 250 rpm, and 0.15 mL of this suspension was spread onto the referred culture media [15]. Concerning settled dust from bakeries (mainly flour), a solution of 0.9% NaCl with 0.05% Tween80™ (Merck S.A., Lisbon, Portugal) was added in a ratio of 4.4 g of settled dust for 40 mL of solution, and 0.15 mL was seeded onto MEA and DG18 [15].

After the incubation of MEA and DG18 at 27°C , for 5 to 7 days, *Aspergillus* sections were identified microscopically, using tease mount or Scotch tape mount and lactophenol cotton blue mount procedures [38]. Morphological identification was achieved through macro- and microscopic characteristics, as noted by De Hoog et al. [38].

2.3. Statistical Analysis

Data were analyzed by using the statistical software SPSS V26.0 for Windows. Results were considered significant at 5% significance level. For the characterization of the sample, n and percentage were used. To study the relationship between the obtained *Aspergillus* sections and the two culture media (MEA and DG18), sampling method applied (active and passive), and the indoor environment assessed, the Chi-Square test was used or, alternatively, the Chi-Square test by Monte Carlo simulation when the applicability assumptions of the Chi-Square test were not verified. In addition to the Chi-Square test, Multiple Correspondence Analysis was used.

Aspergillus sections *Terrei*, *Nidulantes*, *Clavati*, *Usti*, *Cremei*, and *Restricti* presented a weak expression regarding prevalence. Thus, it was considered a group that was called “others”, to allow for the statistical analyses.

3. Results

Overall, total *Aspergillus* counts were higher in MEA ($n = 617$, 53.5%) than in DG18 ($n = 536$, 46.5%). The most frequently identified sections were *Nigri* ($n = 295$, 25.6%), *Fumigati* ($n = 258$, 22.3%), *Circumdati* ($n = 157$, 13.6%), *Versicolores* ($n = 118$, 10.1%), *Candidi* ($n = 115$, 10%), and *Aspergilli* ($n = 102$, 8.8%). The sampling approach and the studied indoor environments may have influenced these data, as it was observed that samples from air ($n = 325$, 28.2%), FRPD ($n = 280$, 24.3%), EDC ($n = 167$, 14.5%), and MPG ($n = 125$, 10.8%) were the ones with higher *Aspergillus* counts. Waste-sorting plants ($n = 483$, 41.9%), health-care facilities ($n = 231$, 20%), and dwellings ($n = 199$, 17.3%) were the indoor environments with higher *Aspergillus* counts (Table 2).

Table 2. Frequency distribution of the detected *Aspergillus* by sections, sampling method, and indoor environment.

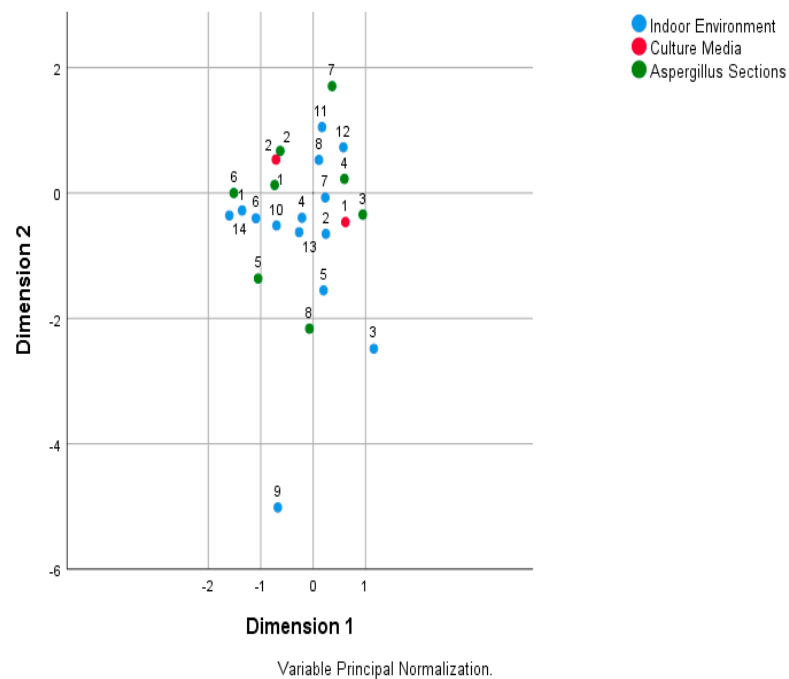
<i>Aspergillus</i> Sections	Media		Sampling Approach	n (%)	Indoor Environment	n (%)
	MEA	DG18				
	n (%)	n (%)				
<i>Candidi</i>	36 (5.8%)	79 (14.7%)	EDC	167 (14.5%)	Bakeries	91 (7.9%)
<i>Circumdati</i>	40 (6.5%)	117 (21.8%)	Air impaction	325 (28.2%)	Dwellings	199 (17.3%)
<i>Nigri</i>	246 (39.9%)	49 (9.1%)	Settled dust	48 (4.2%)	Air	8 (0.7%)
<i>Fumigati</i>	163 (26.4%)	95 (17.7%)	HVAC filter	31 (2.7%)	Taxi	17 (1.5%)
<i>Versicolores</i>	55 (8.9%)	62 (11.6%)	Surface swab	42 (3.6%)	College indoors	17 (1.5%)
<i>Aspergilli</i>	22 (3.6%)	80 (14.9%)	Air impingement	6 (0.5%)	Swine	26 (2.3%)
<i>Terrei</i>	2 (0.2%)	0 (0.0%)	Filter air sampler	90 (7.8%)	Dairies	29 (2.5%)
<i>Flavi</i>	35 (5.7)	48 (9.0%)	MPG	125 (10.8%)	Veterinary clinics	6 (0.5%)
<i>Nidulantes</i>	5 (0.8%)	1 (0.2%)	FRPD	280 (24.3%)	Elementary school	2 (0.2%)
<i>Clavati</i>	3 (0.5%)	2 (0.4%)	Ventilation grid—swab	39 (3.4%)	Healthcare facility	231 (20.0%)
<i>Usti</i>	2 (0.3%)	2 (0.4%)			Elderly care center	11 (1.0%)
<i>Cremeri</i>	1 (0.2%)	0 (0.0%)			Waste sorting	483 (41.9%)
<i>Restricti</i>	7 (1.1%)	1 (0.2%)			Thermal baths	17 (1.5%)
					Firefighters' ambulances	16 (1.4%)
Total	617	536	Total	1153	Total	1153

MEA, malt extract agar; DG18, dichloran-glycerol agar.

Regarding *Aspergillus* sections, significant associations were detected with the media ($\chi^2(7) = 241.118, p < 0.001$), the sampling approach ($p < 0.001, 95\% \text{ C.I.} = (0.3 \times 10^{-4})$), and the indoor environment ($p = 1.5 \times 10^{-4}, 95\% \text{ C.I.} = (0.3 \times 10^{-4})$).

The multiple correspondences analysis was carried out in two parts, as there was a strong relationship between the sampling approach and the indoor environment, and the interpretation was more challenging to achieve. Figure 1 shows the relationship between *Aspergillus* sections, culture media, and indoor environment, where it can be seen that: (i) *Candidi* is associated to the DG18 and the indoor environments swine, healthcare facility, and taxi; (ii) *Circumdati* is associated to the DG18; (iii) *Nigri* is associated to the MEA and to the indoor environment dwellings and dairies; (iv) *Fumigati* is associated to the MEA and to the indoor environments waste-sorting plants, veterinary clinics, and dairies; (v) *Aspergilli* is associated to the indoor environment firefighters' ambulances, bakeries, and swine and to the DG18; (vi) *Flavi* is associated to the indoor environment elderly care center and waste-sorting plant; (vii) others (*Terrei*, *Nidulantes*, *Clavati*, *Usti*, *Cremeri*, and *Restricti*) are associated to the indoor environment college.

Figure 2 shows the relationship between *Aspergillus* sections, media, and sampling approach, where the following can be seen: (i) *Candidi*, *Circumdati*, and *Aspergilli* are associated to the DG18 media; (ii) *Fumigati* and *Flavi* are associated to the sampling approach FRPD; (iii) *Nigri* is associated to the MEA and to the sampling approach ventilation grid—swabs and filter air sampler; (iv) others (*Terrei*, *Nidulantes*, *Clavati*, *Usti*, *Cremeri*, and *Restricti*) are associated to the sampling approach EDC and surface swabs; and (v) *Versicolores* is associated to the sampling approach surface swab.

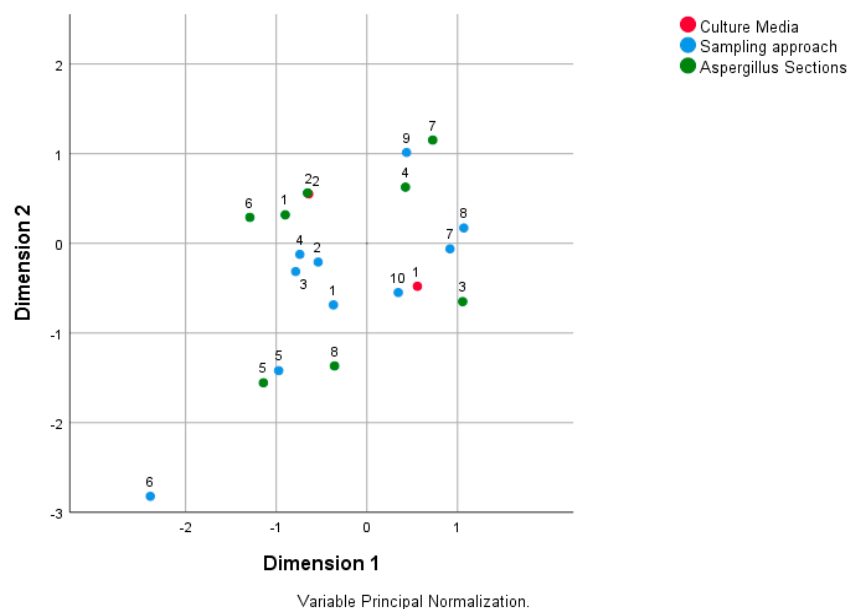


● **Aspergillus sections:** 1 – *Candidi*, 2 – *Circumdati*, 3 – *Nigri*, 4 – *Fumigati*, 5 – *Versicolores*, 6 – *Aspergilli*, 7 – *Flavi*, 8 – Other

● **Media:** 1- MEA, 2 – DG18

● **Indoor Environment:** 1 – Bakeries, 2 – Dwellings, 3 – Air, 4 – Taxi, 5 – Faculty, 6 – Swine, 7 – Dairies, 8 – Veterinary clinics, 9 – Elementary school, 10 – Health care facility, 11 – Elderly care centre, 12 – Waste sorting, 13 – Thermal bath, 14 – Fire-fighters ambulances

Figure 1. Joint plot of category points for *Aspergillus* sections, culture media, and indoor environment.



● **Aspergillus sections:** 1 – *Candidi*, 2 – *Circumdati*, 3 – *Nigri*, 4 – *Fumigati*, 5 – *Versicolores*, 6 – *Aspergilli*, 7 – *Flavi*, 8 – Others

● **Media:** 1- MEA, 2 – DG18

● **Sampling approach:** 1 – EDC, 2 – Air, 3 – Settled dust, 4 – HVAC filter, 5 – Surface swabs, 6 – Air impingment, 7 – Filter air sampler, 8 – MPG, 9 – FRPD, 10 – Ventilation grid - swabs

Figure 2. Joint plot of category points for *Aspergillus* sections, culture media, and sampling approach.

4. Discussion

The aims of any exposure assessment on culturable fungi is to avoid overgrowth of culture plates by fast-growing species, and also to obtain as many different species as possible recovered from the assessed environment [27]. Thus, besides the field (for instance number of liters of air collected) and laboratory (serial dilutions) procedures to avoid the overgrowth, the selection of the culture media is critical to the pursuit of an accurate exposure assessment [13]. Furthermore, in different exposure assessments targeting culturable fungi, the trend was to observe higher counts on MEA and higher diversity on DG18 [13,27,30,31]. However, the studies performed until now have compared the results from both culture media, using the same sampling approach and the same indoor environment.

Aspergillus sections detected in a specific environment depend on three studied variables (culture media, sampling approach, and indoor environment assessment), reinforcing the importance of having scientific data about the background of fungal contamination to select the most suitable protocol to follow from the field to the lab bench [12]. In previous studies, protocols were suggested in order to assess fungal contamination with the following aims: (i) to understand *Aspergillus* occupational exposure assessment [12], (ii) to verify the compliance of a proper indoor air quality [13], and (iii) to obtain information about the *Aspergillus* azole resistance rates in occupational environments with expected azole pressure [14]. In all of those approaches, both culture media (MEA and DG18) were used and all different results were obtained. Taking that into account, but also understanding that a targeted approach should be defined and that resources should be saved, this study was planned to design a proper strategy on samples' culture, depending on the used sampling method and the studied indoor environment.

In this study, the *Versicolores* section was still considered as an independent section. However, this section was recently integrated into the *Nidulantes* section. These taxonomic changes were considered after a performing polyphasic approach to these groups of fungi. In fact, they occur after deep analysis to colonies' morphology, DNA sequencing of specific genes followed by phylogenetic analysis, and observation of different physiological features, including extrolites' production [39,40].

The obtained data revealed that some *Aspergillus* sections were associated with DG18 (*Candidi*, *Circumdati*, and *Aspergilli*) and the indoor environment (*Candidi* and *Aspergilli*), while some were associated with MEA, besides being associated with the sampling approach as well (*Nigri* and *Fumigati*). DG18 inhibits the rapid spreading and restricts the colony size of fast growing genera [41,42], enabling fungal growth of different species [13], including fungal species with clinical and toxigenic relevance, such as specific *Aspergillus* sections as *Fumigati*.

Other sections detected in lower frequency were more frequently associated with the sampling approach (passive sampling methods) and with the indoor environment (*Versicolores*, *Flavi*, *Terrei*, *Nidulantes*, *Clavati*, *Usti*, *Cremeri*, and *Restricti*). Results obtained with passive sampling methods, such as surface swabs and EDC, seem to present a wider diversity of fungal species when compared with the ones obtained by active methods [13,15,43]. Both methods are able to collect data regarding contamination from a longer period of time (from a work shift, days, weeks, or months), while active methods (air sampling) can only reflect the load of a shorter period (mostly minutes) corresponding to the sampling duration [13–15,44].

The EDC is a passive collection device that consists of an electrostatic polypropylene cloth [45]. This standardized method [46] is increasingly being used due to its low cost and efficacy at collecting dust [37,47], and it has already been applied for the occupational exposure assessment of fungal burden [13,15,37,46,48–52]. Thus, sampling with EDC allows a reliable estimation of fungal exposure, since a single EDC analysis is equal to the sum of several air-impaction measurements [53]. Additionally, we should expect the exclusive identification of some fungal species and higher fungal diversity in EDC, when compared to air samples, since the same trend was observed with surface swab samples in previous reports performed in different indoor environments [37,43,54]. Furthermore,

lower contaminated environments (college) tend to have a more diversified fungal flora than the most contaminated indoor environment (waste-sorting plant), where most of the times the dominance of one fungal species indicates an increased health risk [55]. In non-problematic indoor environments, three or more genera should be found, and a dominant genus should not be observed [56].

Both MEA and DG18 can provide useful information regarding the *Aspergillus* genera. In the case of an exploratory exposure assessment study (to determine total *Aspergillus* counts), any of these can be used, or both (ideally). In fact, for the *Aspergillus* total counts at the genus level, results were different between the two culture media, depending on the analyzed indoor environment and on the sampling method used for collection [13,14,16]. The same tendency was observed in this study regarding *Aspergillus* sections. Therefore, in a specific scenario of targeted collection (towards a specific *Aspergillus* section, using a specific collecting approach and/or in a specific indoor environment), a decision regarding the choice of MEA or DG18 should be taken into account (Table 3). This information should be considered in a sampling strategy, to assess a particular *Aspergillus* section, using a defined sampling approach in a specific occupational environment.

Table 3. Information to be considered in a sampling strategy targeting specific *Aspergillus* sections.

Targeted <i>Aspergillus</i> Sections	Culture Media to Use	Indoor/Occupational Environment	Sampling Approach
<i>Aspergilli</i>	DG18	Firefighters' ambulances, bakeries, and swine	*
<i>Candidi</i>	DG18	Swine, healthcare facility, and taxi	*
<i>Circumdati</i>	DG18	*	*
<i>Flavi</i>	*	Elderly care center and waste-sorting plant	FRPD
<i>Fumigati</i>	MEA	Waste-sorting plant, veterinary clinics, and dairies	FRPD
<i>Nigri</i>	MEA	Dwellings and dairies	Ventilation grid—swabs, and filter air sampler
<i>Versicolores</i>	*	*	Surface swabs
<i>Terrei, Nidulantes, Clavati, Usti, Cremei, Restricti</i>	*	College	EDC and surface swabs

* Without association.

5. Conclusions

In light of our results, MEA or DG18 should be selected by taking into consideration the indoor occupational environmental to be assessed, the sampling approach applied, and also the *Aspergillus* section targeted. This study claims attention for the importance to accurately defining the sampling approach and the culture media applied in exposure assessment in a specific indoor environment, when targeted to *Aspergillus*. This information is critical when the exposure assessment needs to target specific *Aspergillus* sections and should be considered in field- and in bench-work protocols. As such, exposure assessors (industrial hygienists, public health officers, IAQ assessors, and laboratory technicians) should be aware of the importance of this information when planning sampling campaigns and the lab work needed.

Author Contributions: Conceptualization, C.V.; methodology, C.V., E.C., and R.S.; validation, C.V. and R.S.; formal analysis, C.V., M.D., and E.C.; investigation, C.V., M.D., and E.C.; resources, C.V.; writing—original draft preparation, C.V., M.D., and E.C.; writing—review and editing, C.V. and R.S.; supervision, C.V.; project administration, C.V.; funding acquisition, C.V. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by FCT—Fundação para Ciência e Tecnologia for funding the project EXPOsE—Establishing protocols to assess occupational exposure to microbiota in clinical settings (02/SAICT/2016—Project n° 23222) and Instituto Politécnico de Lisboa, Lisbon, Portugal for funding the Project “Waste Workers’ Exposure to Bio-burden through Filtering Respiratory Protective Devices” (IPL/2018/WasteFRPD_ESTeSL) and the Project “Occupational exposure of ambulance drivers to bioburden” (IPL/2020/BIO-AmbuDrivers_ESTeSL).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: H&TRC authors gratefully acknowledge the FCT/MCTES national support through the UIDB/05608/2020 and UIDP/05608/2020.

Conflicts of Interest: The authors declare no conflict of interest.

References

- Hernandez, H.; Martinez, L.R. Relationship of environmental disturbances and the infectious potential of fungi. *Microbiology* **2018**, *164*, 233–241. [[CrossRef](#)] [[PubMed](#)]
- Li, Y.; Leung, G.M.; Tang, J.W.; Yang, X.; Chao, C.Y.H.; Lin, J.Z.; Lu, J.W.; Nielsen, P.V.; Niu, J.; Qian, H.; et al. Role of ventilation in airborne transmission of infectious agents in the built environment—A multidisciplinary systematic review. *Indoor Air* **2007**, *17*, 2–18. [[CrossRef](#)] [[PubMed](#)]
- Eames, I.; Tang, J.W.; Li, Y.; Wilson, P. Airborne transmission of disease in hospitals. *J. R. Soc. Interface* **2009**, *6*, S697–S702. [[CrossRef](#)] [[PubMed](#)]
- Douwes, J. (1–3)-Beta-D-glucans and respiratory health: A review of the scientific evidence. *Indoor Air* **2005**, *15*, 160–169. [[CrossRef](#)]
- Eduard, W.; Heederik, D.; Duchaine, C.; Green, B.J. Bioaerosol exposure assessment in the workplace: The past, present and recent advances. *J. Environ. Monit.* **2012**, *14*, 334–339. [[CrossRef](#)]
- Heederik, D.; Von Mutius, E. Does diversity of environmental microbial exposure matter for the occurrence of allergy and asthma? *J. Allergy Clin. Immunol.* **2012**, *130*, 44–50. [[CrossRef](#)]
- Sabino, R.; Veríssimo, C.; Viegas, C.; Viegas, S.; Brandão, J.; Alves-Correia, M.; Borrego, L.M.; Clemons, K.V.; Stevens, D.A.; Richardson, M. The role of occupational *Aspergillus* exposure in the development of diseases. *Med. Mycol.* **2019**, *57*, S196–S205. [[CrossRef](#)]
- Skórska, C.; Sitkowska, J.; Kryszka-Traczyk, E.; Cholewa, G.; Dutkiewicz, J. Exposure to airborne microorganisms, dust and endotoxin during processing of valerian roots on farms. *Ann. Agric. Environ. Med.* **2005**, *12*, 119–126.
- Zielinska-Jankiewicz, K.; Kozajda, A.; Piotrowka, M.; Szadkowska-Stanczyk, I. Microbiological contamination with moulds in work environment in libraries and archive storage facilities. *Ann. Agric. Environ. Med.* **2008**, *15*, 71–78.
- Straumfors, A.; Corbin, M.; McLean, D.; Mannetje, A.; Olsen, R.; Afanou, A.; Daae, H.L.; Skare, Ø.; Ulvestad, B.; Johnsen, H.L.; et al. Exposure Determinants of Wood Dust, Microbial Components, Resin Acids and Terpenes in the Saw- and Planer Mill Industry. *Ann. Work Expo. Health* **2020**, wxz096. [[CrossRef](#)]
- Viegas, C.; Carolino, E.; Sabino, R.; Viegas, S.; Veríssimo, C. Fungal Contamination in Swine: A Potential Occupational Health Threat. *J. Toxicol. Environ. Health Part A* **2013**, *76*, 272–280. [[CrossRef](#)] [[PubMed](#)]
- Viegas, C.; Faria, T.; Aranha Caetano, L.; Carolino, E.; Quintal Gomes, A.; Viegas, S. *Aspergillus* spp. prevalence in different occupational settings. *J. Occup. Environ. Hyg.* **2017**, *14*, 771–785. [[CrossRef](#)] [[PubMed](#)]
- Viegas, C.; Almeida, B.; Monteiro, A.; Aranha Caetano, L.; Carolino, E.; Quintal-Gomes, A.; Twarużek, M.; Kosicki, R.; Marchand, G.; Viegas, S. Bioburden in healthcare centers: Is the compliance with Portuguese legislation enough to prevent and control infection? *Build. Environ.* **2019**, *160*, 106226. [[CrossRef](#)]
- Viegas, C.; Almeida, B.; Aranha Caetano, L.; Afanou, A.; Straumfors, A.; Veríssimo, C.; Gonçalves, P.; Sabino, R. Algorithm to assess the presence of *Aspergillus fumigatus* resistant strains: The case of Norwegian sawmills. *Int. J. Environ. Health Res.* **2020**. [[CrossRef](#)]
- Viegas, C.; Fleming, G.; Kadir, A.; Almeida, B.; Aranha Caetano, L.; Quintal Gomes, A.; Twarużek, M.; Kosicki, R.; Viegas, S.; Coggins, A.M. Occupational exposures to organic dust in Irish bakeries and a pizzeria restaurant. *Microorganisms* **2020**, *8*, 118. [[CrossRef](#)]
- Viegas, C.; Twarużek, M.; Dias, M.; Almeida, B.; Carolino, E.; Kosicki, R.; Soszczyńska, E.; Grajewski, J.; Aranha Caetano, L.; Viegas, S. Assessment of the microbial contamination of mechanical protection gloves used on waste sorting industry: A contribution for the risk characterization. *Environ. Res.* **2020**, *189*, 109881. [[CrossRef](#)]
- Madsen, A.M.; Frederiksen, M.W.; Jacobsen, M.H.; Tendal, K. Towards a risk evaluation of workers’ exposure to handborne and airborne microbial species as exemplified with waste collection workers. *Environ. Res.* **2020**, *183*, 109177. [[CrossRef](#)]
- Varga, J.; Baranyi, N.; Chandrasekaran, M.; Vágvölgyi, C.; Kocsibé, S. Mycotoxin producers in the *Aspergillus* genus: An update. *Acta Biol. Szeged.* **2015**, *59*, 151–167.
- Jeanvoine, A.; Rocchi, S.; Reboux, G.; Crini, N.; Crini, G.; Millon, L. Azole-resistant *Aspergillus fumigatus* in sawmills of Eastern France. *J. Appl. Microbiol.* **2017**, *123*, 172–184. [[CrossRef](#)]

20. Snelders, E.; van der Lee, H.; Kuijpers, J.; Rijs, A.J.M.M.; Varga, J.; Samson, R.A.; Mellado, E.; Donders, A.R.T.; Melchers, W.J.G.; Verweij, P.E. Emergence of azole resistance in *A. fumigatus* and spread of a single resistance mechanism. *PLoS Med.* **2008**, *5*, e219. [\[CrossRef\]](#)
21. Chowdhary, A.; Kathuria, S.; Xu, J.; Meis, J. Emergence of azole-resistant *Aspergillus fumigatus* strains due to agricultural azole use creates an increasing threat to human health. *PLoS Pathog.* **2013**, *9*, e1003633. [\[CrossRef\]](#)
22. Verweij, P.E.; Kema, G.H.J.; Zwaan, B.; Melchers, W.J.G. Triazole fungicides and the selection of resistance to medical triazoles in the opportunistic mould *Aspergillus fumigatus*. *Pest Manag. Sci.* **2013**, *69*, 165–170. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Dillon, H.K.; Boling, D.K.; Miller, J.D. Comparison of detection methods for *Aspergillus fumigatus* in environmental air samples in an occupational environment. *J. Occup. Environ. Hyg.* **2007**, *4*, 509–513. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Burge, H.A.; Otten, J.A. Chapter 19 Fungi. In *Bioaerosols: Assessment and Control*; Macher, J., Ed.; American Conference of Governmental Industrial Hygienists: Cincinnati, OH, USA, 1999; Volume 19, pp. 1–13.
25. Viegas, C.; Viegas, S.; Gomes, A.; Täubel, M.; Sabino, R. (Eds.) *Exposure to Microbiological Agents in Indoor and Occupational Environments*; Springer: Berlin/Heidelberg, Germany, 2017; pp. 109–128.
26. Burge, H.A.; Chatigny, M.; Feeley, J.; Kreiss, K.; Morey, P.; Otten, J.; Peterson, K. Guidelines for assessment and sampling of saprophytic bioaerosols in the indoor environment. *Appl. Ind. Hyg.* **1987**, *2*, R10–R16.
27. Wu, P.; Su, H.J.; Ho, H. A comparison of sampling media for environmental viable fungi collected in a hospital environment. *Environ. Res.* **2000**, *82*, 253–257. [\[CrossRef\]](#)
28. Hung, L.L.; Miller, J.D.; Dillon, H.K. *Field Guide for the Determination of Biological Contaminants in Environmental Samples*, 2nd ed.; American Industrial Hygiene Association: Fairfax, VA, USA, 2005; pp. 93–100.
29. Agência Portuguesa para o Ambiente. *Qualidade do Ar Interior—Um Guia Técnico*; Agência Portuguesa para o Ambiente: Amadora, Portugal, 2009.
30. Smid, T.; Schokkin, E.; Boleij, J.S.M.; Heederik, D. Enumeration of viable fungi in occupational environments: A comparison of samplers and media. *Am. Ind. Hyg. Assoc. J.* **1989**, *50*, 235–239. [\[CrossRef\]](#)
31. Verhoeff, A.P.; Van Wijnen, J.H.; Boleij, J.S.M.; Brunekreef, B.; Van Reenen-Hoekstra, E.S.; Samson, R.A. Enumeration and identification of airborne viable mould propagules in houses: A field comparison of selected techniques. *Allergy* **1990**, *45*, 275–284. [\[CrossRef\]](#)
32. Chao, H.J.; Milton, D.K.; Schwartz, J.; Burge, H.A. Dustborne fungi in large office buildings. *Mycopathologia* **2002**, *154*, 93–106. [\[CrossRef\]](#)
33. Degois, J.; Clerc, F.; Simon, X.; Bontemps, C.; Leblond, P.; Duquenne, P. First Metagenomic Survey of the Microbial Diversity in Bioaerosols Emitted in Waste Sorting Plants. *Ann. Work Expo. Health* **2017**, 1–11. [\[CrossRef\]](#)
34. Croston, T.L.; Nayak, A.P.; Lemons, A.R.; Goldsmith, W.; Gu, J.K.; Germolec, D.R.; Beezhold, D.H.; Green, B.J. Influence of *Aspergillus fumigatus* conidia viability on murine pulmonary 28 micro RNA and m RNA expression following subchronic inhalation exposure. *Clin. Exp. Allergy* **2016**, *46*, 1315–1327. [\[CrossRef\]](#)
35. Timm, M.; Madsen, A.M.; Hansen, J.V.; Moesby, L.; Hansen, E.W. Assessment of the total inflammatory potential of bioaerosols by using a granulocyte assay. *Appl. Environ. Microbiol.* **2009**, *75*, 7655–7662. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Viegas, S.; Aranha Caetano, L.; Korkalainen, L.; Faria, T.; Pacífico, C.; Carolino, E.; Quintal Gomes, A.; Viegas, C. Cytotoxic and inflammatory potential of air samples from occupational settings with exposure to organic dust. *Toxics* **2017**, *5*, 8. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Viegas, C.; Faria, T.; Monteiro, A.; Aranha Caetano, L.; Carolino, E.; Quintal Gomes, A.; Viegas, S. A Novel Multi-Approach Protocol for the Characterization of Occupational Exposure to Organic Dust—Swine Production Case Study. *Toxics* **2018**, *6*, 5. [\[CrossRef\]](#) [\[PubMed\]](#)
38. De Hoog, D.; Guarro, J.; Gene, G.; Figueras, M. *Atlas of Clinical Fungi—The Ultimate Bench Tool for Diagnosis, Version 4.1.4*; Utr. Centraalbureau voor Schimmelcultures: Utrecht, The Netherlands, 2016.
39. Chen, A.J.; Frisvad, J.C.; Sun, B.D.; Varga, J.; Kocsubé, S.; Dijksterhuis, J.; Kim, D.H.; Hong, S.B.; Houbaken, J.; Samson, R.A. *Aspergillus* section *Nidulantes* (formerly *Emericella*): Polyphasic taxonomy, chemistry and biology. *Stud. Mycol.* **2016**, *84*, 1–118. [\[CrossRef\]](#)
40. Samson, R.A.; Visagie, C.M.; Houbaken, J.; Hong, S.-B.; Hubka, V.; Klaassen, C.H.W.; Perrone, G.; Seifert, K.A.; Susca, A.; Tanney, J.B.; et al. Phylogeny, identification and nomenclature of the genus *Aspergillus*. *Stud. Mycol.* **2014**, *78*, 141–173. [\[CrossRef\]](#)
41. Bergwall, C.; Stehn, B. Comparison of selective mycological agar media for the isolation and enumeration of xerophilic moulds and osmotolerant yeasts in granulated white sugar. *Zuckerindustrie* **2002**, *127*, 259–264.
42. Corry, J.E.L.; Curtis, G.D.W.; Baird, R.M. *Handbook of Culture Media for Food and Water Microbiology*, 3rd ed.; Royal Society of Chemistry: London, UK, 2011; ISBN 1847551459, 9781847551450.
43. Viegas, C.; Faria, T.; Meneses, M.; Carolino, E.; Viegas, S.; Quintal Gomes, A.; Sabino, R. Analysis of Surfaces for Characterization of Fungal Burden—Does it Matter? *Int. J. Occup. Med. Environ. Health* **2016**, *29*, 623–632. [\[CrossRef\]](#)
44. Badyda, A.; Gayer, A.; Czechowski, P.; Majewski, G.; Dąbrowiecki, P. Pulmonary function and incidence of selected respiratory diseases depending on the exposure to ambient PM10. *Int. J. Mol. Sci.* **2016**, *17*, 1954. [\[CrossRef\]](#)
45. American Conference of Governmental Industrial Hygienists (ACGIH). *Threshold Limit Values for Chemical Substances and Physical Agents and Biological Exposure Indices*; American Conference of Governmental Industrial Hygienists: Cincinnati, OH, USA, 2009.

46. Cozen, W.; Avol, E.; Diaz-Sanchez, D.; McConnell, R.; Gauderman, W.J.; Cockburn, M.G.; Mack, T.M. Use of an electrostatic dust cloth for self-administered home allergen collection. *Twin Res. Hum. Genet.* **2008**, *11*, 150–155. [[CrossRef](#)]
47. Kilburg-Basnyat, B.; Metwali, N.; Thorne, P.S. Performance of electrostatic dust collectors (EDCs) for endotoxin assessment in homes: Effect of mailing, placement, heating and electrostatic charge. *J. Occup. Environ. Hyg.* **2016**, *13*, 85–93. [[CrossRef](#)]
48. Noss, I.; Wouters, I.M.; Visser, M.; Heederik, D.J.J.; Thorne, P.S.; Brunekreef, B.; Doekes, G. Evaluation of a low-cost electrostatic dust fall collector for indoor air endotoxin exposure assessment. *Appl. Environ. Microbiol.* **2008**, *74*, 5621–5627. [[CrossRef](#)] [[PubMed](#)]
49. Normand, A.C.; Vacheyrou, M.; Sudre, B.; Heederik, D.J.J.; Piarroux, R. Assessment of dust sampling methods for the study of cultivable microorganism exposure in stables. *Appl. Environ. Microbiol.* **2009**, *75*, 7617–7623. [[CrossRef](#)] [[PubMed](#)]
50. Madsen, A.M.; Matthiesen, C.B.; Frederiksen, M.W.; Frederiksen, M.; Frankel, M.; Spilak, M.; Timm, M. Sampling, extraction and measurement of bacteria, endotoxin, fungi and inflammatory potential of settling indoor dust. *J. Environ. Monit.* **2012**, *14*, 3230–3239. [[CrossRef](#)] [[PubMed](#)]
51. Dorado-Garcia, A.; Bos, M.E.; Graveland, H.; Van Cleef, B.A.; Verstappen, K.M.; Kluytmans, J.A.; Wagenaar, J.A.; Heederik, D.J. Risk factors for persistence of livestock-associated MRSA and environmental exposure in veal calf farmers and their family members: An observational longitudinal study. *BMJ Open* **2013**, *3*, e003272. [[CrossRef](#)] [[PubMed](#)]
52. Feld, L.; Bay, H.; Angen, Ø.; Larsen, A.R.; Madsen, A.M. Survival of LA-MRSA in dust from swine farms. *Ann. Work Expo. Health* **2018**, *62*, 147–156. [[CrossRef](#)] [[PubMed](#)]
53. Institute of Medicine. *Damp Indoor Spaces and Health*; The National Academies Press: Washington, DC, USA, 2004.
54. Cabo Verde, S.; Almeida, S.M.; Matos, J.; Guerreiro, D.; Meneses, M.; Faria, T.; Botelho, D.; Santos, M.; Viegas, C. Microbiological assessment of indoor air quality at diferente hospital sites. *Res. Microbiol.* **2015**, *166*, 557–563. [[CrossRef](#)]
55. Hicks, J.B.; Lu, E.T.; De Guzman, R.; Weingart, M. Fungal types and concentrations from settled dust in normal residences. *J. Occup. Environ. Hyg.* **2005**, *2*, 481–492. [[CrossRef](#)]
56. Hodgson, M.; Scott, R. Prevalence of fungi in carpet dust samples. In *Bioaerosols, Fungi and Mycotoxins: Health Effects, Assessment, Prevention and Control*; Johanning, E., Ed.; Boyd Printing Company: Albany, NY, USA, 1999; pp. 268–274.